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<b>(54) Title:</b> VACCINE PRODUCTION OF THE <i>BACILLUS ANTHRACIS</i> PROTECTIVE ANTIGEN		
<b>(57) Abstract</b>  Methods of preparing recombinant <i>Bacillus anthracis</i> protective antigen or a variant or fragment thereof for use in vaccines is disclosed. The protein is expressed in a recombinant microorganism which comprises a sequence which encodes PA or said variant or fragment thereof wherein either (i) a gene of the microorganism which encodes a catabolic repressor protein and/or AbrB is inactivated, and/or (ii) wherein a region of the PA sequence which can act as a catabolic repressor binding site and/or an AbrB binding site is inactivated. Useful quantities of protein are obtainable from these organisms.		

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VACCINE PRODUCTION OF THE *BACILLUS ANTHRACIS* PROTECTIVE ANTIGEN

5 The present invention relates to the production of immunogenic proteins such as the protective antigen (PA) of *Bacillus anthracis* using recombinant DNA technology, to expression vectors and hosts used in the production process and to methods of their preparation.

10

*Bacillus anthracis*, the causative agent of anthrax possesses two main virulence factors, a poly-D-glutamic capsule and a tripartite protein toxin. PA, the non-toxic, cell-binding component of the toxin, is the  
15 essential component of the currently available human vaccine. The vaccine is usually produced from batch cultures of the Sterne strain of *B. anthracis*, which although avirulent, is still required to be handled as a Class III pathogen. In addition to PA, the vaccine  
20 contains small amounts of the anthrax toxin moieties, edema factor and lethal factor, and a range of culture derived proteins. All these factors contribute to the recorded reactogenicity of the vaccine in some individuals. The vaccine is expensive and requires a six  
25 month course of four vaccinations. Furthermore, present evidence suggests that this vaccine may not be effective against inhalation challenge with certain strains (M.G. Broster et al., Proceedings of the International Workshop on Anthrax, 11-13 April 1989, Winchester UK. Salisbury  
30 med Bull Suppl No 68, (1990) 91-92).

Previous workers have attempted to produce PA in *Escherichia coli* (M.H. Vodkin et al., Cell, (1983) 34, 693-697) and *Salmonella typhimurium* (N.M. Coulson et al.,  
35 Vaccine (1994) 12, 1395-1401) but for reasons which are

not known, the level of production of PA was low in these organisms.

*B. subtilis* is a harmless bacterium usually found in the  
5 environment. The possibility of using a genetically  
transformed *B. subtilis* to produce just PA, without  
other, undesirable components of the anthrax toxin, and  
without the need for rigorous containment has previously  
10 been proposed (B.E. Ivins et al., Infection and Immunity  
(1986), 54, 537-542). In particular, the gene encoding  
the protective antigen moiety of the tripartite exotoxin  
of *B. anthracis* was cloned into *B. subtilis* IS53 using  
the plasmid vector pUB110. Two clones, PA1 and PA2, were  
15 obtained, both of which produced more PA in liquid  
cultures than the Sterne strain of *B. anthracis* with  
levels of up to 41.9 mg/l being achieved. However, the  
organism also produced proteolytic enzymes, albeit in low  
quantities, which degraded the PA and made subsequent  
purification difficult.

20 This PA expression system (*B. subtilis* IS53(pPA102)) has  
been evaluated (L.W.J. Baillie et al., Lett Appl.  
Microbiol. (1994) 19, 225-227). The system suffered from  
a down-regulation of the PA gene in early fermentation  
25 and was not proposed for large-scale production of PA  
antigen.

For production on an industrial scale, for example in  
vaccine production, it is important to maximise yields of  
30 protein for cost reasons. It is also helpful to obtain  
protein in the form of full length protein as this will  
be easier to purify than a selection of proteolytic  
fragments. The applicants have identified a number of  
expression factors which lead to improved levels of PA  
35 production.

Hence the present invention provides a recombinant microorganism which is able to express *Bacillus anthracis* protective antigen or a variant or fragment thereof which is able to generate an immune response in a mammal, said  
5 microorganism comprising a sequence which encodes PA or said variant or fragment thereof wherein either (i) a gene of said microorganism which encodes a catabolic repressor protein and/or AbrB is inactivated, and/or (ii) a region of the said PA sequence which can act as a  
10 catabolic repressor binding site is inactivated; and/or (iii) a region of the said PA sequence which can act as an AbrB binding site is inactivated.

Variants and fragments of PA must be able to produce an  
15 immune response in a mammal to whom they are administered. The immune response is suitably protective against infection by *Bacillus anthracis* although the protective effect may be seen only after repeated applications, as would be determinable by methods known  
20 in the art. Variants comprise peptides and proteins which resemble PA in their effect, but have different amino acid sequence. For example, variants may be 60% homologous to PA protein, suitably 80% homologous and more particularly at least 90% homologous. Fragments are  
25 suitably peptides which contain at least one antigenic determinant of PA, or variants thereof.

As used herein, the expression "functional equivalent" refers to moieties such as nucleotide sequences or  
30 proteins, which although different to the reference moieties in certain respects, qualitatively fulfill the same biological function.

A suitable microorganism for use as a host organism is a  
35 strain of *Bacillus subtilis*. Suitable strains are available from various sources including the *Bacillus*

Genetic Stock Center, The Ohio State University, Columbus, Ohio, USA from where strains such as IA147 and IA172 may be obtained. Additional strains are described in the literature, for example by Perego et al.,

5 Molecular Microbiology, (1988) 2, 689-699 where strains JH642 and JH12575 are described.

Preferably however, the microorganism of the invention comprises a strain which produces little or no proteases,

10 since PA is very susceptible to decomposition by protease. A particularly preferred strain of *Bacillus subtilis* is a protease deficient strain. One such strain is *B. subtilis* WB600. This organism has been engineered to be deficient in six extracellular proteases

15 (Xu-Chu Wu et al., J. Bacteriol. (1991) 173, 4952-4958). This strain is able to produce high yields of PA, for example of up to 40mg/l which allowed the development of a purification strategy.

20 Catabolite repression of gene expression involves the trans-acting factors Catabolite control protein A (CcpA) and the phosphocarrier protein Hpr (Saier et al., Microbiology (1996), 142, 217-230). It has been proposed that CcpA binds to a catabolite-responsive

25 element sequence in the control region of catabolite-sensitive operons and prevents transcription when glucose is present (Henkin et al. Molecular Microbiology (1991) 5, 575-584).

30 AbrB is a transition state regulator which prevents inappropriate gene expression during vegetative growth. Like CcpA, AbrB binds to DNA and prevents gene transcription (Strauch et al., J. of Bacteriology (1995), 177, 6999-7002).

Comparison of the level of PA expression from wildtype and mutant strains revealed that PA is subject to catabolite repression and AbrB regulation. In particular, it was found that PA levels from pPA101-1 are  
5 three fold higher in a *ccpA* mutant than in an otherwise isogenic parent, and eight fold higher in an *abrB* mutant. Thus, the introduction of mutations affecting catabolite repression and growth phase regulation into strains which are not deficient in these may result in an increase in  
10 the yield of PA in this host-vector system.

Screening of the PA control region for potential catabolite repressor binding sites revealed a region with 81% homology which started 37 bases downstream of the  
15 translational start point (see Figure 3 hereinafter). Screening with the *abrB* consensus sequence produced three regions which showed between 82-89% homology. The closest match was for a region which included the P2 translational start point and overlapped the ribosome  
20 binding site. Thus PA repression may be due to Catabolite control protein A (*CcpA*) and *AbrB* binding directly to these target sequences.

Suitably therefore, one or both of these sites are  
25 inactivated so as to increase the expression of PA. Inactivation may be effected by for example by mutation of the relevant site. The skilled person would be able to produce these, for example using site directed mutagenesis, and test for the required inactivation using  
30 routine techniques.

Preferably however, these activities are inhibited by inactivation of the gene which produce the relevant proteins (e.g. *AbrB* or *CcpA*). Either a host strain which  
35 is deficient in the genes which produce either or both of these proteins are employed, or one or both of the genes

of the host strain are inactivated. Suitable inactivation techniques include insertion mutagenesis, where preferably a selection marker gene is inserted into the relevant gene in the host DNA using conventional methods. A suitable selection marker gene is Tn917 which encodes the antibiotic marker erythromycin.

Catabolite repressor activity may additionally or alternatively be inhibited by controlling the growth media in which the organisms are cultured. This control may be effected by excluding certain sugars such as glucose which invoke this activity, and using alternative carbon sources such as glycerol and such methods form a further aspect of the invention.

*Bacillus anthracis* protective antigen may be obtained by culturing a recombinant microorganism as described above and such a process forms a further aspect of the invention. In a preferred embodiment, the microorganisms of the invention are cultured under conditions in which catabolite repressor activity is minimised as described above.

The repression of gene expression by amino acid mixtures has been described (Atkinson et al., Journal of Bacteriology, (1990) 172, 4758-4765). Although the mechanism of this repression is not yet fully understood, recent work has shown that a DNA binding protein called CodY is involved in the amino acid repression of a number of genes (Serror et al. Proceedings of the 8th International Conference on Bacilli, (1995) July 8-12, Stanford, USA, p39. There is some evidence from growth studies with *B.anthraxis* to suggest that PA expression may be subject to amino acid repression (Bartkus et al., Infection and Immunity, (1989) 57, 2295-2300; S.H.Leppla,



SourceBook of Bacterial Toxins, ed. J.E.Alouf and J.H.Freer, pp277-302, Academic Press).

5 The amino acid composition of the media has been found to influence the level of PA expression and therefore the organism is preferably cultured in the presence of at least one amino acid which stimulates PA expression. The levels of said amino acid may be boosted as desired by addition of the amino acid to the culture media. Using a  
10 prototrophic variant of a particular strain of *B. subtilis* (*B. subtilis* WB600pPA101-1), alanine stimulated PA expression (whilst tryptophan inhibited it).

15 In addition, since catabolite repressor activity appears to be important, the organism is suitably cultured in a medium which lacks sugars such as glucose, which invoke this activity. A preferred carbon source is glycerol.

20 Recombinant microorganisms as described above may be prepared using conventional technology. The desired nucleic acid sequences may be incorporated into one or more suitable expression vectors and these vectors used to transform a host strain, in particular a prokaryotic host such as *B. subtilis*.

25 For example, the available plasmid vector pUB110 may be used to clone the gene encoding PA into a strain of *B. subtilis* as described for example by B. E Ivins et al., supra., and the resultant strain further modified as  
30 described above.

Nucleotide sequences prepared and vectors for use in this process form a further aspect of the invention.

The invention will now be particularly described by way of example with reference to the accompanying drawings in which:

5 Figure 1 illustrates the structure of pPA101-1;

Figure 2 shows a partial sequence of the PA gene; and

Figure 3 shows the results of PA expression from the pag  
10 gene in pPA101-1 in different genetic backgrounds.

In the following Examples, the strains and plasmids referred to are shown in Table 1.

Table 1

<u>Strain/plasmid</u>	<u>Phenotype</u>	<u>Reference/Source</u>
<u>B. subtilis</u>		
WB600	trpC2, ΔnpreE, Δapre EΔepr, bpf, mpr::ble , nprB::ery	Xu-Chu Wu et al., (1991) supra.
IA 147	alsA1*, alsR1, ilvΔ1, trpC2	BGSC†
IA172	ilvΔ1, trpC2	BGSC
JH642	trpC2, pheA1	Perego et al (1988) supra.
JH12575	trpC2, pheA1, abrB::Tn917	Perego et al (1988) supra.
<u>Plasmids</u>		
pPA101	Km <sup>r</sup> ; PA‡	Ivins et al., (1986) supra
pPA102	Km <sup>r</sup> ; PA <sup>*</sup>	Ivins et al., (1986) supra
pPA101-1	Km <sup>r</sup> ; PA <sup>*</sup>	Example 1 below

5

\* *alsA* and *ccpA* genes are allelic (Henkin et al.,  
Molecular Microbiology, (1991) 5, 575-584)

† *Bacillus* Genetic Stock Center, The Ohio State  
10 University, Columbus, Ohio, USA.

‡ Protective Antigen

## Example 1

Production of Plasmid pPA101-1

A new plasmid pPA101-1 was derived from pPA101 following  
5 transformation into *B. subtilis* WB600 using the protoplast  
method of Chang and Cohen (MGG (1979) 168, 111-115) with  
appropriate antibody selection. Plasmids were isolated  
from transformants and purified for sequencing using the  
QIAGEN plasmid purification kit (QIAGEN Inc. Chatsworth,  
10 USA).

Comparison of the restriction maps of pPA101 (Ivins et  
al. 1986, supra.) show that approximately 1.7kb of DNA,  
from the vector and the 5' region of the PA-containing  
15 insert, had been deleted resulting in a plasmid similar  
in size to pPA102(6.1kb)

Nucleotide sequencing was performed by cycle sequencing  
in a Catalyst Molecular Biology LabStation using a ABI  
20 PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit  
with AmpliTaq Polymerase FS. This was followed by  
electrophoresis in a 373A DNA Sequencing System (Applied  
Biosystems). Sequence data were analysed using the  
EditSeq programme option of the DNASTAR Inc computer  
25 package (Abacus House, West Ealing, London, W13 0AS).

The published PA gene sequence (Welkos et al. Gene (1988)  
69, 287-300) was used to design sequencing primers. The  
sequence determined in one strand was fully overlapped.  
30 When ambiguities occurred, they were resolved by  
sequencing the complementary strand.

This exercise revealed that the sequence of the vector/PA  
insert junction regions of pPA101-1 and pPA102 were  
35 identical. Analysis of this junction region showed the  
presence of a single copy of a 5 base sequence, TCTAT,

which has been shown previously to occur in both pUB110 (complement of positions 1838-1842) and in the PA sequence (positions 1640-1644) (Figure 1). The sequences flanking this junction corresponds to those expected if the DNA between the two TCTAT sequences had been deleted.

The sequence encoding the PA protein in pPA101-1 differed in only one base from the published sequence. Using the numbering system of Welkos et al., 1988 supra, a single base change was found at positions 2743 (G to C) which would change a GAA codon to a CAA codon (glutamic acid to glutamine).

The original PA-encoding clones pPA101 and pPA102 are based on the plasmid pUB110. This plasmid replicates via a single-stranded dexoyribonucleic acid intermediate by a rolling-circle replication mechanism (Gruss et al. Microbiological Reviews (1989) 53, 231-241). Such plasmids are particularly prone to deletion events such as homologous recombination between relatively short repeats (3 to 13 bases), which can result in the loss of several thousand bases (Ehrlich et al. Genetic Engineering, ed. J.K. Setlow et al. (1986) vol. 8, p71-83 Plenum publishing Corp., New York). Although both plasmids contained deletions, the junction points between vector/insert had not been defined. The data here suggest that pPA101-1, which was originally derived from pPA101, may have arisen as a consequence of recombination events between two TCTA sequences, leading to its present form which appears to be similar to pPA102.

#### Example 2

##### Effect of an *abrB* mutation on PA expression

Strains JH12575 (*AbrB*<sup>-</sup>) and JH642(*AbrB*<sup>-</sup>) were transformed with pPA 101-1 using the protoplast method of Chang and

Cohen (MGG (1979) 168, 111-115). The transformed strains were then grown.

Culture conditions and media for AbrB repression studies have been described previously (L.W.J.Baillie et al., Proceedings of the International Workshop on Anthrax 19-21 Sept 1995, Winchester, UK. Salisbury Med. Bull. 1996 87 (special suppl.) 133-135. Briefly 100 ml of culture medium in a 250ml screw-capped Duran bottle was inoculated with 0.1ml of a suspension of the organism in saline ( $OD_{540}$  1.3). A rich culture medium based on yeast extract and tryptone was employed (Fahnestock et al., J. Bacteriol. (1986), 165,1011-1014). Specifically the medium contained per litre of deionised water, Bacto yeast extract (Difco) (20g), Tryptone (Difco) 33g, NaCl 7.4g  $Na_2PO_4$  8g,  $KH_2PO_4$  4g and L-histidine 1g. The pH was adjusted to 7.4 and the medium was sterilised by autoclaving at 115°C for 15 minutes. The medium in which plasmid-containing strains were cultured was supplemented with the appropriate antibiotic (kanamycin - final concentration 10mg l<sup>-1</sup>).

Cultures were incubated with shaking (150 rev min<sup>-1</sup>) at 37°C. The progress of growth was monitored by optical density ( $OD_{540}$ ) and was continued until the cultures reached late log/stationary phase growth ( $OD_{540}$  of 1) at which time a sample of culture supernatant was assayed for PA.

The resultant solutions were assayed for PA by ELISA as described by Ivins et al. (1986) supra.. The initial binding antibodies were rabbit polyclonal anti-PA. Human polyclonal anti-PA IgG was used to bind captured PA. PA/antibody complex was detected using goat anti-human horse radish peroxidase conjugate (Sera-Lab Ltd, Sussex), and developed with 2,2' azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) substrate.

PA values for the individual strains were determined as follows: cultures were grown to an  $OD_{550}$  of approx. 1. Culture supernatants were assayed for PA by ELISA and the data normalised to a culture  $OD_{550}$  of 1.0. Due to the possibility of plate to plate variation and a shortage of purified reference PA, the PA values for each mutant and its otherwise isogenic parent were determined on the same ELISA plate. Mean values were based on 8 or more assays per sample. The non-specific background mean, measured in an otherwise isogenic strain lacking the PA gene, was then subtracted. The data were analysed statistically and the results are illustrated in Figure 3 section (a).

It is clear that 8-fold more PA ( $P < 0.05$ , mean of the two determinations) was produced from the *abrB* strain.

### Example 3

#### Effect of a *ccpA* mutation on PA expression

Following the procedures described in Example 2 above, pPA101-1 was transformed into a *CcpA* strain, IA147, and the otherwise isogenic parent strain IA172. The culture process and analysis were similar to those described in Example 2 except that the medium formulated for this study contained per litre of deionised water, Tryptone (Difco) 20g,  $K_2HPO_4$  (7g),  $KH_2PO_4$  (3g),  $(NH_4)_2SO_4$  (1g), sodium citrate (0.5g),  $MgSO_4 \cdot 7H_2O$  (100mg) and L-histidine 1g. The pH was adjusted to 7.4 and the medium was sterilised by autoclaving at 115°C for 15 minutes. Following autoclaving sterile glucose was added to a concentration of 2%.

The results are shown in Figure 3b and illustrate that in a *CcpA* background and in a tryptone based medium, there

was a three-fold increase in the level of PA expression ( $P < 0.05$ , mean of two determinations).

#### Example 4

##### 5 Effect of amino acids on PA expression

To study the effect of amino acids on PA expression from a prototrophic strain of *B. subtilis* WB600 p101-1, the organism was grown in basal minimal medium (BMM) supplemented with tryptophan (150mg/ml) and in BMM  
10 supplemented with a 16 amino acid mixture (M.R. Atkinson et al., J. Bacteriol (1990) 172, 4758-4765).

The BMM contained per litre of deionised water:  $K_2HPO_4$  (7g),  $KH_2PO_4$  (3g), sodium citrate (0.5g)  $MgSO_4 \cdot 7H_2O$   
15 (100mg),  $(NH_4)_2SO_4$  (2g), glycerol to 2% (w/v), L-histidine (1g), uracil (1.4mg), adenine sulphate (2.1mg), thiamine HCl (1mg),  $CaCl_2$  (10mg),  $FeSO_4 \cdot 7H_2O$  (2.5mg),  $ZnSO_4 \cdot 7H_2O$  (2.5mg),  $MnSO_4 \cdot 3H_2O$  (2.5mg) and  $H_2SO_4$  (0.1N). The pH was adjusted to 7.4 and the medium was filter sterilised  
20 using a 0.2µm filter (German Sciences, Ann Arbor, Michigan, USA).

The 16 amino acid mixture, which was added to the BMM where required contained L-cysteine (40mg l<sup>-1</sup>), L-arginine  
25 HCl (400mg l<sup>-1</sup>), L-isoleucine (200mg l<sup>-1</sup>), L-valine (200mg l<sup>-1</sup>), L-glutamate (800mg l<sup>-1</sup>), L-lysine (100mg l<sup>-1</sup>), L-phenylalanine (100mg l<sup>-1</sup>), L-proline (100mg l<sup>-1</sup>), L-threonine (100mg l<sup>-1</sup>), L-aspartate (665mg l<sup>-1</sup>), L-alanine (445mg l<sup>-1</sup>), glycine (375mg l<sup>-1</sup>), L-serine (525mg l<sup>-1</sup>), L-tryptophan  
30 (150mg l<sup>-1</sup>), L-methionine (160mg l<sup>-1</sup>) all from filter sterilised stocks.

The culture conditions for PA expression were as follows: 100ml of medium in a 250ml screw-capped Duran bottle was  
35 inoculated with 0.1ml of a suspension of the organism in saline ( $OD_{550}$  1.3). Cultures were incubated with shaking



(150 rev min<sup>-1</sup>) at 37°C. The progress of growth, monitored by optical density (OD<sub>540</sub>, was continued until the cultures reached late log/stationary phase growth at which time a sample of culture supernatant was assayed for PA. When  
5 plasmid-containing strains were cultured the medium was supplemented with the appropriate antibiotic: kanamycin (final concentration 10mg/l).

The time course of PA expression was determined by taking  
10 samples at hourly intervals during the course of culture and assaying them for PA. Samples were assayed for PA by ELISA as described in Example 2 above.

The PA content of the culture supernatant was determined  
15 by ELISA and the specific activity (mean ELISA-determine PS absorbance, A<sub>412</sub>, per optical density unit (OD<sub>540</sub>) was determined.

The results showed that there was a 3.6 fold reduction  
20 (p>0.5, mean of two determinations) in the level of PA expression when the organism was grown in the presence of the amino acid mixture.

#### Example 5

##### 25 Nucleotide sequence search for negative regulator binding sites

A region of the published nucleotide sequence of PA from base 1600 to 2000 which contains the promoter region and the first 195 bases of the structural gene was screened  
30 for homology with the consensus sequence, TGWNANCGNTNWCA, which codes for the catabolite repressor operator (M.J. Wieckert et al., Proc. Natl. Acad. Sci. USA (1990) 78, 6238-6242) and WAWWTTTWCAAAAAAW, a 16bp consensus sequence based on 20 observed AbrB binding regions (M.A.  
35 Strauch et al., J. Bacteriol. (1995) 177, 6999-7002).

This programme allows a selected sequence to be searched for a particular site pattern or matrix sequence.

This resulted in the identification of sites which are homologous to the catabolite repressor binding site as well as the *abrB* binding site as illustrated by underlining in Figure 3.

Hence inhibition of catabolite repressor protein and/or *AbrB* within the organism, for example by insertion mutagenesis of the gene encoding these proteins, or by adjusting the media accordingly, or by mutation of the binding sites as outlined hereinbefore would increase the yield of PA.

15

#### Example 6

##### Purification and efficacy of recombinant PA

Recombinant protective antigen (rPA) was purified to homogeneity from *Bacillus subtilis* using the following method.

20

rPA was fractionated from cell culture supernatant with ammonium sulphate followed by ion exchange chromatography on FPLC MonoQ HR 10/10 and finally gel filtration chromatography on FPLC Superose 10/30, yielding 7mg rPA per litre culture. Homogeneous recombinant PA was characterised in terms of native and subunit molecular weight, and isoelectric point.

25

The protective efficacy of rPA against airborne challenge with the AMES strain of *B. anthracis* was determined in the presence of the adjuvants: Alhydrogel and RIBI. Maximum protection was achieved when rPA was adjuvanted with RIBI, in the guinea pig model.

35

## Claims

- 5 1. A recombinant microorganism which is able to express *Bacillus anthracis* protective antigen or a variant or fragment thereof which is able to generate an immune response in a mammal, said microorganism comprising a sequence which encodes PA or said variant or fragment  
10 thereof wherein either (i) a gene of said microorganism which encodes a catabolic repressor protein and/or AbrB is inactivated, and/or (ii) wherein a region of the said PA sequence which can act as a catabolic repressor binding site is inactivated; and/or (iii) a region of the  
15 said PA sequence which can act as an AbrB binding site is inactivated.
2. A microorganism according to any claim 1 which comprises a recombinant strain of *Bacillus subtilis*.  
20
3. A microorganism according to claim 1 or claim 2 wherein a gene which encodes a catabolic repressor protein and/or AbrB is inactivated by insertion mutagenesis.  
25
4. A microorganism according to claim 5 wherein the inserted sequence comprises a selection marker gene.
5. A microorganism according to claim 6 wherein the  
30 selection marker gene is Tn917.
6. A microorganism according to claim 1 which comprises a CcpA or an AbrB mutant strain.

7. A microorganism according to claim 1 wherein a region of the said PA sequence which can act as a catabolic repressor binding site is inactivated.
- 5 8. A microorganism according to claim 3 wherein said catabolic repressor binding site comprises a region of the PA gene located between bases 1842-1854 as shown in Figure 2.
- 10 9. A microorganism according to claim 1 wherein a region of the PA sequence which can act as an AbrB binding site is inactivated.
- 15 10. A microorganism according to claim 9 wherein said AbrB binding site comprises a region of the PA gene located between bases 1778-1792 as shown in Figure 2.
- 20 11. A microorganism according to any one of claims 7 to 10 wherein inactivation of catabolic repressor binding site and/ or an AbrB binding site is effected by mutation of said site.
- 25 12. A process for producing *Bacillus anthracis* protective antigen or an immunogenic variant or fragment thereof, which process comprises culturing a recombinant microorganism according to any one of the preceding claims and recovering PA therefrom.
- 30 13. A process according to claim 12 wherein the organism is cultured in the presence of at least one amino acid which stimulates PA expression.
14. A process according to claim 13 wherein said amino acid is alanine.

15. A process according to any one of claims 12 to 14 wherein the microorganism is cultured in the presence of glycerol and in the substantial absence of glucose.

5 16. A process for producing PA or an immunogenic variant or fragment thereof, which process comprises culturing a microorganism which is able to express PA or said variant or fragment thereof in the presence of a medium which favours PA expression.

10

17. A process according to claim 16 wherein said conditions comprise the absence of a carbon source which invokes catabolite repressor activity.

15 18. A process according to claim 16 or claim 17 wherein glycerol is used as the carbon source in the culture medium.

19. A process for preparing a recombinant  
20 microorganism according to any one of claims 1 to 11 which process comprises transforming a microorganism with a vector comprising a sequence encoding PA or an immunogenic variant or fragment thereof wherein either  
(i) a gene which encodes a catabolic repressor protein  
25 and/or AbrB in said microorganism is inactivated; and/or  
(iii) a region which can act as a catabolic repressor binding site and/or a region which can act as an AbrB binding site in the said sequence encoding PA or an immunogenic fragment or variant thereof is inactivated

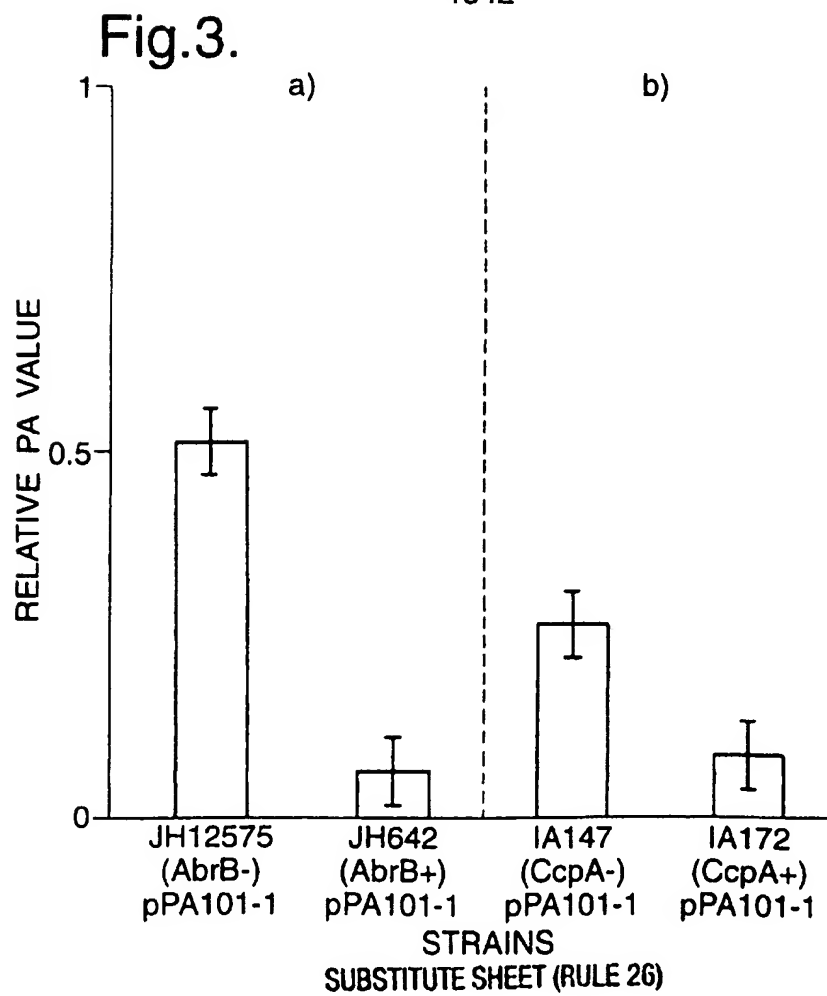
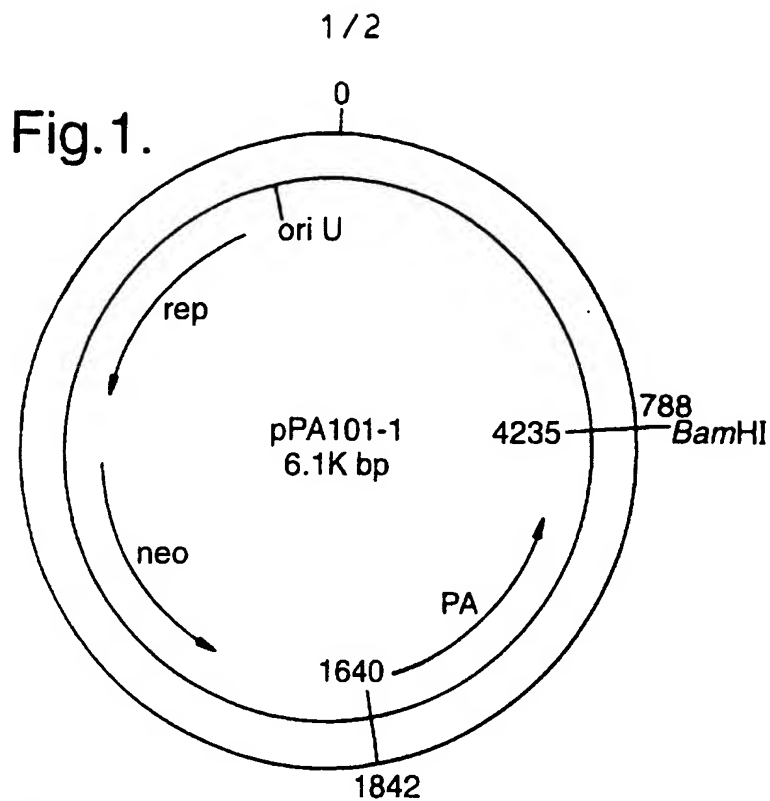
30

20. A novel vector for use in the process of claim 19.

21. A nucleotide sequence for inclusion in the vector of claim 17.

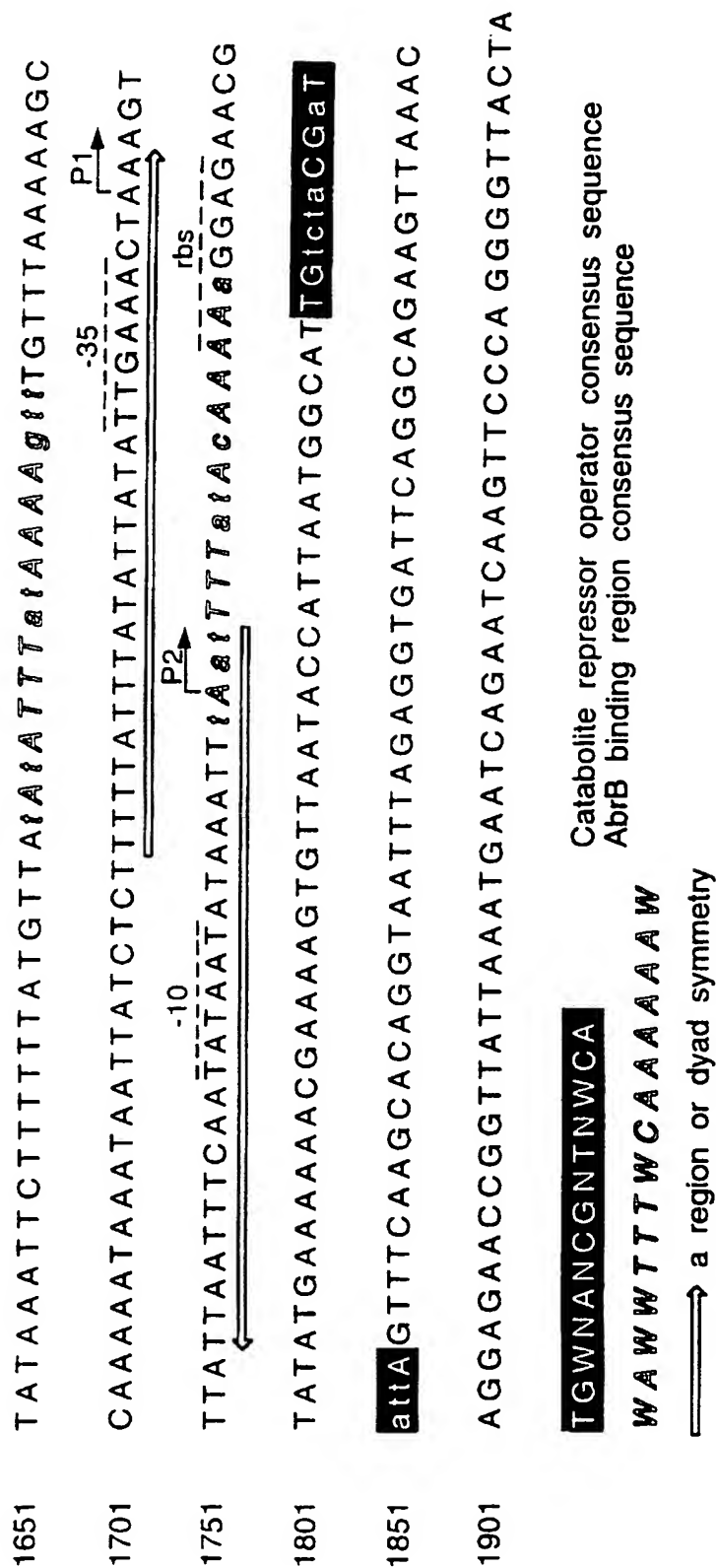
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22. Bacillus anthracis protective antigen or an immunogenic variant or fragment thereof obtainable by a method according to any one of claims 13 to 18.



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Fig.2.





<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/31 C12N15/75 C12N1/21 C07K14/32 A61K39/07 //(C12N1/21,C12R1:125)		
According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STRAUCH MA: "AbrB modulates expression and catabolite repression of a Bacillus subtilis ribose transport operon." J BACTERIOL, DEC 1995, 177 (23) P6727-31, UNITED STATES, XP002049153 see the whole document ---	1-13, 15-22
Y	HUECK CJ ET AL: "ANALYSIS OF A CIS-ACTIVE SEQUENCE MEDIATING CATABOLITE REPRESSION IN GRAM-POSITIVE BACTERIA" RESEARCH IN MICROBIOLOGY, 1994, 145, 503-518, XP002049154 see the whole document --- -/--	1-13, 15-22
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex.		
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Date of the actual completion of the international search		Date of mailing of the international search report
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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Gurdjian, D

## INTERNATIONAL SEARCH REPORT

International Application No

PC1/GB 97/02288

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	IVINS BE ET AL: "Cloning and expression of the Bacillus anthracis protective antigen gene in Bacillus subtilis." INFECT IMMUN, NOV 1986, 54 (2) P537-42, UNITED STATES, XP002049155 see the whole document ---	1-13, 15-22
A	MIWA Y ET AL: "DETERMINATION OF THE CIS SEQUENCE INVOLVED IN CATABOLITE REPRESSION OF THE BACILLUS-SUBTILIS GNT OPERON IMPLICATION OF A CONSENSUS SEQUENCE IN CATABOLITE REPRESSION IN THE GENUS BACILLUS" NUCLEIC ACIDS RESEARCH, 18 (23). 1990. 7049-7054., XP002049156 see the whole document ---	1-13, 15-22
T	KRAUS A ET AL: "Analysis of CcpA mutations defective in carbon catabolite repression in Bacillus megaterium." FEMS MICROBIOL LETT, AUG 1 1997, 153 (1) P221-6, NETHERLANDS, XP002049157 see the whole document -----	1-13, 15-22

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